Role of *EGLN1* Genetic Polymorphism in Viral Infections

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Certificate

I hereby declare that the short synopsis Title "**Role of *EGLN1* Genetic Polymorphism in Viral Infections**" being submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy in **Department of Biotechnology** under School of Engineering and Technology of Manav Rachna International Institute of Research & Studies, Faridabad during the academic year 2024-2025. It is a bona fide record of original work carried out under the supervision of Dr. Kanchan Bhardwaj and has not been submitted in full or part to any other university or institute for degree or diploma. Further, it is certified that short synopsis is plagiarism free, and proof has been submitted.

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ABSTRACT

Tibetans expressing PHD2$^{D4E;C127S}$ barely display any physiological response to hypoxia at high altitude that are typical of acclimatization and mountain sickness. The enzymatic activity of PHD2 is driven by binding of oxygen. Active Prolyl Hydroxylase 2(PHD2) hydroxylates HIF α subunits creating a binding site for the protein Von Hippel-Lindau resulting in its polyubiquitination and proteasomal degradation under normoxia. Under hypoxia, as PHD2 enzyme cannot bind to enough oxygen, HIF α is stabilized. This results in increase of erythropoiesis and facilitates pathogenesis of Acute Mountain Sickness. The Tibetan haplotype of *EGLN1* expressing PHD2 $^{D4E;C127S}$ has 30% lower Km value for oxygen than PHD2^{WT} help them to adapt in hypoxic environment.

To check the role of PHD2 $^{D4E;C127S}$ in viral susceptibility we have infected U937-monocytic and Huh7-liver cell line expressing PHD2 $^{D4E;C127S}$ with different RNA viruses like Dengue, Japanese Encephalitis and SARS-CoV-2. We have checked their viral genome copy, viral protein-NS1, NS3, Spike, Interferon α, Interferon β and Interferon related genes like *IRF3*, *IRF7*, *IRF9* under normoxia and hypoxia(3%). They are susceptible to viral infections when they are at sea level (normoxic condition) whereas interestingly they become less prone under hypoxic environment. We will validate this finding by using Primary monocytes expressing PHD2$^{D4E;C127S}$ from Tibetan individuals.

Keywords: Tibetans, Polymorphism, PHD2, HIF α, *EGLN1*

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1. INTRODUCTION

High-altitude environments display distinctive challenges to individuals. At elevations, because of reduced atmospheric pressure leads to lowering the oxygen tension. At elevations above 2,500 meters, the reduced oxygen availability may lead to exert profound physiological complications.[1] Sojourners at high altitudes may experience hypoxic symptoms such as headache, nausea, vomiting and fatigue. These hypoxic sickness are collectively called as acute mountain sickness.[2] Persistence AMS can results in more critical conditions high-altitude pulmonary oedema (HAPE) and high-altitude cerebral oedema (HACE), impacting the respiratory and central nervous systems.[3]The pathophysiology includes impaired oxygen sensing ability which results in cerebral vasodilation, impaired pulmonary artery pressure and increased permeability of the capillary and blood-brain barrier.[4-5] Hypobaric hypoxia triggers hypoxia-induced polycythemia. Due to hypoxiation, erythropoietin, released by kidney, eliciting bone marrow to generate more RBCs, erythrocytosis to enhance the oxygen-carrying capacity. This orchestrated response helps in oxygen transport to different organs and tissues. However, excess RBCs lead to AMS.[6-7]

Though high altitude is defined as complex and harsh condition, human populations have come up with these challenges and residing there. The key mechanism behind this high-altitude adaptation is genetic polymorphisms, particularly single nucleotide polymorphisms (SNPs). Three high altitude dwellers, Ethiopians, Andeans, and Tibetans exhibit distinct genetic adaptations to thrive in oxygen-deprived environments.[8-10] HIF2A protein is encoded by *EPAS1*. This endothelial PAS domain-containing protein 1 (*EPAS1*) gene play a pivotal role in oxygen homeostasis in hypoxic adaptation. Notable variant rs13419896 of *EPAS1* is associated with elevated hemoglobin levels, enhancing oxygen-carrying capacity, tissue oxygenation and oxygen transport. Another gene *EGLN1* has remarkable role in reducing the detrimental effects of high-altitude hypoxia.[11-16] Genomic landscape of Tibetans revealed genetic variants associated with hypoxic regulation in *EPAS1* gene are rs12374818 and rs17039192 and in *EGLN1* is rs186996510.[17-19] Prolyl hydroxylase domain 2(PHD2) is an oxygen sensor enzyme, encoded by *EGLN1* gene, regulates cellular response to hypoxia modulating the central transcription factor, hypoxia-inducible factor(HIF).[20-21] Under normoxic condition, PHD2 hydroxylates proline residues at sites 402 and 564 of the oxygen dependent domain of HIF-1α, making binding sites for protein von Hippel-Lindau (VHL) leading to ubiquitination and subsequent proteasomal degradation of HIF-α. However, under low oxygen condition PHD2 cannot bind with oxygen, thus HIF-α is stabilized. HIF-α binds with HIF-β forming

dimer in the nucleus, activate different hypoxia regulatory elements/genes (HREs) 5'-RCGTG-3' related with erythropoiesis, angiogenesis, and oxygen homeostasis.[22-24] Tibetans exhibit a high frequency missense polymorphism in the *EGLN1* gene c[12C>G; 380G>C] which determines the adaptive response at high altitude. This variant having 30% low Km value for oxygen helps in degradation of HIFs.[15] PHD2 hydroxylates proline residues of P-AKT leading to suppression of its kinase activity.[25-26] AKT is also known as Protein kinase B. It is serine/threonine kinase involved in different cellular process such as cell survival, cell metabolism, cell growth and proliferation. When AKT gets phosphorylated, its kinase activity gets activated, this P-AKT plays crucial role in cellular functions. After entering cells, virus manipulates cellular signaling pathways to improve their replication by inhibiting apoptosis[27] enhancing their entry[28], modulating host immune response[29] and promoting cell survival and proliferation[30].

Hypoxic environment directly affects immune cell functions, pro-inflammatory and anti-inflammatory cytokine production, connected with immunosuppression which affects both innate and adaptive immune response. Hypoxia can change the phenotype and activity of different immune cells such as neutrophils, macrophages, monocytes, and T cells[31-33]. Under hypoxic conditions, monocytes activated by HMGB1 secrete pro-inflammatory cytokines but not type I interferons. HIF1α is the direct transcription suppressor of Interferon regulatory factors (*IRF5*, *IRF3*) in monocytes leading to suppress interferon production under hypoxia[34]. HIF1α plays an important role in regulating IFNγ inducible genes, highlighting IFNγ-dependent immunity against infection[35]. Both acute and chronic hypoxic conditions, CD3+ T cells are decreased thus entirely reduction of CD4+ T cells. A significant reduction has been found in expression of IFNγ in T cells[36].

We have investigated the role of Tibetan specific PHD2$^{D4E;C127S}$ polymorphisms in viral infections under hypoxia and normoxia. Primary monocytes expressing PHD2$^{D4E;C127S}$ infected with DENV2 showing high replication and reduced interferon response under normoxia but less replication and elevated interferon response under hypoxic conditions. We also checked % HIF1α positive cells and found elevated in PHD2$^{D4E;C127S}$ monocytes under normoxia . However, became decreased under hypoxia. Since having low Km value for oxygen, PHD2$^{D4E;C127S}$ can bind with oxygen and helping in ubiquitination and proteasomal degradation of HIFα under hypoxia, thus protect from hypoxic complications[15]. Elevated HIF1α levels corelates with decreased interferon production in PHD2$^{D4E;C127S}$ monocytes under normoxia and reverse under hypoxia. As, recent studies has shown,

HIF1α is the negative regulator of interferon regulatory factors[35]. To confirm the role of $PHD2^{D4E;C127S}$ in viral infections, in engineered $PHD2^{D4E;C127S}$ and $PHD2^{WT}$ U937 or Huh7 cells infected with DENV2 or SARS-CoV-2 we observed the same phenomenon in vitro. Treatment with HIF1α-inhibitor CAY10585 significantly increased interferon productions and rescued from DENV2/SARS-CoV-2 infections. Expression of P-AKT also observed elevated in SARS-CoV-2 infected $PHD2^{D4E;C127S}$ cells under normoxia. P-AKT inhibitor TCN helps in reducing ACE2 expression in cells thus reduces infection also[37-38]. CD4/CD8 T-cells of $PHD2^{D4E/C127S}$ individuals showed less expression of IFN-γ upon exposure to SARS-CoV-2 RBD peptides under normoxia but increased under hypoxia compared with $PHD2^{WT}$ counterpart.

2. LITERATURE REVIEW AND RESEARCH GAPS

Living at an elevation of 2500m and above is challenging for human beings. Travelers who are not acclimatized to hypobaric hypoxia develop acute mountain sickness. These include vomiting, headache, nausea, pulmonary edema, cerebral edema, loss of appetite, and disturbed sleep. Decreased oxygen percentage at high altitude results in low barometric pressure. At high altitude, hypoxia induces the secretion of pro-inflammatory cytokines IL6, IL8, TNFα and increases vascular permeability.[39] Hypoxia has an immense effect on central nervous system that may results in permanent damage to nervous system. Hypoxia thinners the blood plasma, thus increases the hemoglobin concentration. It can directly stimulate the juxtra-glomerulus region of kidney to secrete erythropoietin. It often gives rise to Acute Mountain Sickness (AMS).[40] AMS is characterized by cerebral edema and pulmonary edema, increased erythrocytosis and hypertension. In pregnant mother, this can restrict the intrauterine growth which leads to prenatal death.[41]

In spite of these challenges, human populations started living at high altitude 200 million years ago. Three high altitude populations, Tibetans, Andeans, and Ethiopians adapt themselves to reside at elevations.[42] In Tibetans, two naturally selected genes *EPAS1* and *EGLN1* help them to adopt at high altitude.[43] Tibetans do not show elevated hemoglobin conc. at high altitude. Single nucleotide polymorphisms have been found that help the Tibetans to adapt under such hypoxic conditions. Naturally selected *EGLN1* gene of chromosome 1 help them to adapt under hypoxic condition. Two missense mutations present at 12^{th} (C>G) and 380^{th} position (G>C). This haplotype is found in Tibetans and Denisovans, data were collected from blood samples of Tibetans of Himalaya plateau. This gene encodes protein called prolyl hydroxylase 2 has affinity for oxygen. This Tibetan variant of PHD2 has high affinity and low KM value for oxygen thus help them under hypobaric hypoxia.[44] Living at >3000 m, Tibetans show distinctive physiological adaptations towards hypobaric hypoxia.[45,46] From genome wide analysis of 3008 Tibetans and 728 non-Tibetans, genotype was studied for a total of 7.3 million SNPs. Naturally selected two genes were found in Tibetans are *MTHR* and *EPAS1*, play important role in hemoglobin function also.[47-49]

Dengue is one of mosquito borne viral infection causes death worldwide. Around 50-105 million people get infected by dengue virus annually, among them almost 21,000 deaths. Dengue virus mainly transmitted by saliva of Aedes mosquito. It belongs to family Flaviviridae. Cases mostly found in tropical and sub-tropical regions of the world. It leads to Dengue Fever and Dengue Shock Syndrome. Till date there is no therapeutic approach of this infection. Its structure is like other

viruses from Flaviviridae family such as Japanese Encephalitis, West Nile, ZIKA, yellow fever virus. It has positive sense single stranded genomic RNA and consists of three structural and seven nonstructural proteins.[50-51]

JEV is one of most important encephalitis disease in India. It mainly affects children. This arboviral disease mainly occur in tropical and sub-tropical regions of the world. It affects the neuronal cells of the brain that leads to malfunction of the brain. Till now five serotypes of JE virus have been reported.[52] In 2019, JEV strain IV 19CxBa-83-Cv have been isolated from pool of mosquitoes in Bali, Indonesia.[53]

SARS-CoV-2 variant is the 3rd highest pathogenic among all from coronavirus family. The Pandemic because of SARS-CoV-2 became great threats to human population. To combat this disease several vaccines and therapeutic approaches have been developed. Several studies have reported the current advances of research, vaccine develop, antiviral compounds, several antibodies derived therapeutic strategies.[54-55]

Chikungunya virus is alpha virus transmitted by mosquitoes. It caused epidemic throughout worldwide among human populations. This can cross placental membrane and cause infection from mother to baby. It also causes musculoskeletal deformities which last for several years. Sometimes it also leads to seronegative rheumatoid arthritis in elder people.[56-57]

SARS-CoV-2 infection affect native highlanders also. Hypobaric hypoxia impacts on the survival of the residents. 1st of SARS-CoV-2 infection case at Peru was reported on 6th March and by three months approx. 224120 positive cases were reported. Among them deaths were 6498. Study reported that less infection at high altitude but not fatality rate is hampered due to high altitude.[58-59] Dengue virus is also a great threat to Brazil.[60] Multicenter cross-sectional study had revealed presence of different DENV serotypes in Nepal. Studies reported presence of DENV NS1, anti-DENV IgG, anti-DENV IgM in the patients from Nepal. DENV I, II and III serotypes were detected but no DENV IV was found in Nepal.[61] Different viral diseases such as Marburg, Ebola, Nipah, West-Nile encephalitis, HIV, Lassa, Dengue, Yellow fever are great threat to human population globally. Now-a-days, chikungunya virus become a great threat. It became endemic in Indian subcontinental region, southeast Asia, Africa, Asia and Europe.[62-63]

Identified Gaps in Literature:

Recently, we have shown that the Tibetan specific mutations of prolyl hydroxylase-2 (PHD2), $PHD2^{D4E/C127S}$ (*EGLN1* gene, c.12C>G and c.380G>C) protects this highlander population form hypoxia-triggered clinical events such as polycythemia and pulmonary/cerebral edema at the high altitude > 3500 meters. Being an oxygen-dependent enzyme PHD2 binds to O_2 and hydroxylates hypoxia inducible factor (HIF)-1α, and in turn destabilizes HIF function. Under hypoxia, PHD2 cannot bind to enough O_2 and therefore stabilizes HIF. This results in an increased erythropoiesis and inflammation, resulting in acute mountain sickness (AMS) among the non-Tibetan Mountain travelers. On the other hand, the Tibetans specific PHD2 $^{D4E;C127S}$ variant has increased affinity (with 30% lower Km) for O_2 than $PHD2^{WT}$, thus suppressing the HIF function under hypoxic environment. This novel genetic adaptation helps Tibetan highlanders to adapt in extreme hypobaric hypoxia.

We have found that primary monocytes as well as monocytic cell line expressing PHD2 $^{D4E;C12S}$ release less pro-inflammatory cytokines TNF, IL-6 and IL-1β than that of wild type PHD2 expressing cells under hypoxia. These cytokines are crucial in eliciting immune response against infections, so it is possible that they might be less susceptible to some infections.

Literature shows at high altitude there is less infection among Tibetan individuals; one reason being isolated and less exposed, but when travelers visit there from sea level, they become high prone to any infection. The Tibetans individuals when come to sea level if they get infected it persists long. What is the underlying mechanism behind this? Is it related to Hypoxia Inducible Pathways or AKT pathways, as Tibetans has polymorphism in their *EGLN1* gene which encodes protein PHD2. PHD2 regulates both HIF and AKT pathways.

3. DESCRIPTION OF BROAD AREA AND PROBLEM STATEMENT

Total percentage of oxygen in air is constant in all altitudes, but due to an increase in the partial pressure, inspired oxygen becomes less and thus the gaseous exchange in the lungs is also reduced. Atmospheric pressure is the sum of pressure exerted by all the gases, including nitrogen, oxygen, carbon-di-oxide, and water vapor (6.3kPa). Oxygen constitutes 0.21% of the total. At sea level, inspired oxygen pressure is 19.6 kPa. At 3000m height, inspired oxygen pressure is 13.3 kPa and alveolar oxygen pressure is only 8 kPa. At the time of tissue injury, the cells experience hypoxic conditions. RBC count decreases below normal range and cardiac output of oxygen becomes less, leading to less oxygen supply to the tissue. Under hypoxic stress, epithelial cells secrete Tumor Necrosis Factor alpha and Interleukin 8. This releases Interleukin 6 leading to increased vascular permeability.[39] Sojourners who travel to high altitude developed AMS, HAPE and have increased pro-inflammatory parameters such as cytokines, NFκB signaling molecules. When sojourners travel to high altitudes, initially for 12-24 hours they usually do not show any symptoms but after that 24 hour, they start showing headaches, nausea, insomnia, breathlessness etc. AMS may not be fatal in most cases but due to excessive breathlessness, after a few days, travelers started showing prolonged cough with white sputum which simultaneously progressed into blood sputum. This is known as High Altitude Pulmonary edema (HAPE). This is due to hypoxia pulmonary vasoconstriction and if not treated, leads to a fatal condition.[40] Vasodilators and calcium antagonists have been used to treat HAPE. Those who have physiological traits like Tibetans, have high oxygen saturation of hemoglobin. Several high-altitude populations have been found all over the world like in Asia (Tibetans), America (Andeans), and Africa (Ethiopians). Physiological adaptations at high altitudes have emerged independently in these populations. For example, in Andean and Tibetan high-altitude populations, oxygenation of arterial hemoglobin is decreased compared to the Ethiopian high-altitude population. Hemoglobin concentration is enhanced in Andeans relative to Ethiopians and Tibetans. Four of the HIF pathway genes, Nitric Oxide Synthetase 2 (*NOS2A*), Endothelin 1(*EDN1*), ADRA1b Alpha-1 adrenergic Receptor (*ADRA1b*), and Prolyl hydroxylase 3 (PHD3) play role in positive selection for high altitude adaptation in Andeans. The other genes that help in physiological adaption are *EGLN1*, Vascular Endothelial Growth Factor (*VEGF*), Cadherin (*CDH1*), Endothelin receptor A (*EDNRA*) and Protein Kinase (*PRKAA1*).[41] The SNPs found responsible for high altitude adaptations in Tibetans are *EGLN1*, *EPAS1*, and Peroxisome Proliferator-Activated Receptor Alpha (*PPARA*). Tibetans having $PHD2^{D4E;C127S}$ variant adapted well to harsh conditions of high altitudes.

Their pro-inflammatory cytokines like IL1β, IL6, signaling molecules -ATF2, RELA, JUN, STAT1 levels were lower compared to wild type (WT) counterparts.[64] αKG is the co-factors of enzyme PHD2. αKG decreases platelet activation, aggregation, and granular secretion. p-AKT and PHD2 interactions was confirmed by immunoprecipitation. Different surface markers of platelets such as P-Selectin, Annexin, PAC-1 are suppressed and decreased microparticle release upon Octyl αKG treatment in a concentration dependent manner. On the other hand, PHD2 inhibitor, DKG activates those above parameters. αKG does not alter PI3K functions which is upstream molecule of AKT. αKG suppresses p-AKT in monocytes isolated from healthy individuals. There is suppression in secretion of inflammatory cytokines like IL1β, IL6, IL10, TNFα in those monocytes treated with αKG. Significant amount of intracellular αKG was found to be increased and decreased succinate in platelets after Octyl αKG treatment. αKG supplementation in mice platelets also showed reduced platelet activation and aggregation. In case of SARS-CoV-2 infection, there is increased inflammation and thrombosis which is rescued by supplementation with Octyl αKG.[65] Studies have reported that supplementation with αKG reduces platelet activation, aggregation, cytokine secretion both in vivo and in vitro and DKG increases those above parameters. αKG reduces tail, lung and liver thrombosis and systemic and lung inflammation. Also, it rescues viral load, lung thrombosis, liver thrombosis in SARS-CoV-2 infected mice.

Plasma leakage and vascular permeability are the one of major hallmarks of the dengue infection. Toll Like Receptor 2 along with its co-receptor TLR6 or CD14 help in recognizing dengue particles and helps dengue virus to penetrate within the host cells. Blocking TLR2, blocks NFκB signaling pathway. On the other hand, blocking TLR6 or CD14 has moderate effect on NFκB. Together innate immune cells, endothelial and epithelial cells are responsible for generating early inflammatory response that prevents the virus from invading the host. Both primary monocytes and monocytic cell line-THP1 (DV2 infected) with DV2-activated platelet and platelet supernatant had higher dengue replication compared to only Dengue infection. Mass spectrometry analysis revealed that seven platelet proteins are highly co-related with dengue virus infection, out of which Platelet Factor 4(PF4) was the highest. When the cells are treated with PF4, the viral infection increases and anti PF4 antibodies reduce it. PF4 inhibits interferon pathways molecules like IRF9 and STAT2. It inhibits interferon production in monocytes. Inflammatory molecules like NFκB, IκBα and cytokines such as TNF α, IL6, IL1β are increased upon PF4 treatment. In dengue patient's samples at 3rd day post infection, where there is elevated PF4 level in plasma, dengue NS1 is also high.[66] PF4 treatment increases JEV replication also. Observations support that the platelet cytokine, PF4 is crucial for

both dengue and JEV replication. It inhibits interferons and targeting PF4 can be used as an antiviral strategy. Dengue or JEV infection leads to UPR (Unfolded Protein Response) activation. This UPR activation induces pro-inflammatory cytokine production and generates Reactive Oxygen Species (ROS) production. Treatment with AMG487 reduced JEV replication in neuro2a, N9 cell line and mice. AMG487 significantly increased body weight of JEV infected mice. Inflammatory parameters such as percentage cellularity, pro-inflammatory cytokines TNFα, IL6, IL1β, and signaling molecules like p-P65, and p-AKT also decreased upon AMG487 treatment. Interferons, IFNα, IFNβ, and inflammatory regulatory genes are up-regulated in AMG487-treated JEV-infected mice. This suggests that the drug improves ROS production and decreases JEV-induced cell death. The UPR pathway genes such as NRF2, ATF6, CHOP, ATF4, spliced XBP1 and IRE1α and decreased upon AMG487 treatment of JEV infected mice group. AMG487 helps in improving body weight, viral replication, inflammation, ROS generation, and UPR activation in JEV-infected mice.[67] Virus activates the AKT signaling pathway through FoxO1 which helps in cell survival. Studies have already reported that AKT signaling has an important role in SARS-CoV-2replication. AKT also has a role in regulating interferon responses and in ACE2 expression. AKT phosphorylation also regulates mTOR, P70S6K translation, and the production of inflammatory cytokines. SARS CoV2-infected Vero cells with αKG and TCN treated showed decreased viral replication. Both αKG and TCN decrease viral replication in transiently ACE2 expressing U937 monocytic cell line also. αKG decreased SARS-CoV-2 infection, lung inflammation, and thrombosis in hamsters. αKG did not alter IFNγ+ T cells rather it helped in reducing the production of pro-inflammatory cytokines and microparticle release from platelets.[68] αKG helped in elevating oxygen saturation in SARS-CoV-2 infected hamsters. SARS-CoV-2 infection is a major concern in the human population. It belongs to the family Beta-coronaviridae. An increased D-dimer and thrombin level in the plasma of the patients. Platelet leukocyte aggregation and platelet microparticle release were also elevated in SARS-CoV-2 infected patients. Platelet transcriptomic analysis from SARS-CoV-2 infected patients has revealed 598 up-regulated transcripts and 676 down-modulated transcripts compared to healthy controls. Pro-inflammatory cytokines and P-p38, P-ERK are elevated in covid patients.[69]

Hypoxic ventilatory response is low in Andeans but no change both in Tibetans and Ethiopians. Arterial oxygen saturation has been elevated in Andeans and Ethiopians but not in Tibetan individuals. Hemoglobin concentration and pulmonary arterial pressure are increased in all three high altitude populations. Birth weight and Nitric oxide are increased both in Andeans and Tibetans, but no change is observed in high altitude Ethiopians populations. Under Normal oxygen condition,

PHD2 hydroxylates HIFα subunit and induces its proteasomal degradation. Hence HIFα subunit cannot bind with HIFβ subunit resulting no translation of hypoxic transcripts. On the other hand, under less oxygen condition, PHD2 cannot bind with oxygen thus cannot induce the degradation of HIFα subunit. Studies have reported genetic alteration of some genes such as PHD2, HIF1α, HIF2α have potential role in regulating high-altitude adaptations.[70]

4. RESEARCH OBJECTIVES

1. To investigate the effect of PHD2$^{D4E;C127S}$ polymorphism on the replication of RNA viruses.
2. To evaluate the Type I Interferon response in virus-infected PHD2^{WT} and PHD2$^{D4E;C127S}$ host cells.
3. To examine the PHD2-regulated pathways, upon infection of PHD2^{WT} and PHD2$^{D4E;C127S}$ cells with RNA viruses.

5. RESEARCH METHODOLOGY

1. **Human Ethics:**

   Human ethics approval was acquired from Institutional Ethics Committee of the Regional Centre for Biotechnology and Sher-i-Kashmir Institute of Medical Sciences to study the human samples. Reference numbers are the followings- RCB-IEC-H-01 and RCB-IEC-H-22. The written consent form was received from all the volunteers.

2. **Study subjects:**

   We collected 5mL of whole blood from Healthy Tibetan Volunteers (without having any history of Chronic diseases, Diabetes, not using drugs for long time, aged between 18-42 years) in citrate dextrose anticoagulant vials. Plasma and PBMC were used for further studies.

3. **Genotyping of SNPs**:

   From whole blood, genomic DNA was isolated using a Qiagen FlexiGene DNA kit (Cat.no-51206). The SNPs of *EGLN1* (NCBI RefSeq NG_015865) are found in exon 1 of chromosome 1. SNPs were amplified by PCR using the primers of *EGLN1*. Phusion Polymerase Master Mix (GC rich) was used. Amplification was executed at 98°C for 5 mins, 98°C for 120 secs (40 cycles), 60.1°C-60 secs, and 72°C- 120 secs, then final extension at 72°C- 5 mins and a 4°C hold. It generated a product size of 1025bps. The amplified DNA was run in 1% agarose gel followed by purified using a Qiagen gel extraction kit (Cat. no-28706) and sequenced to identify the SNPs c.12C>G; 380G>C.[64]

4. **Plasma and PBMC isolation:**

   Whole blood was centrifuged at 500 rpm with acceleration 9 and break free for 15 minutes at room temperature. The upper transparent layer (Platelet Rich Plasma) was collected and subjected to centrifuge again to separate Platelet and Plasma (Platelet Poor Plasma) at 1000g for 15 minutes. The bottom red fraction layer was diluted with 1X PBS at a 1:1 ratio and gently pipette the diluted fraction on top of each 3mL of Lymphoprep, followed by

centrifuging at 550g with acceleration 5 and break free for 26 minutes at room temperature. The middle cloudy layer was collected and washed with 1X PBS.[64]

5. Monocyte isolation:

After isolating PBMCs using Lymphoprep from whole blood by density gradient centrifugation, PBMCs were stained with CD14+ micro-bead and kept for 46 mins at 4 °C temp and monocytes were isolated through MACS column. 0.3×10^6 cells per well CD14+ monocytes were seeded in 12-well cell plates in RPMI-1640 media supplemented with 10% (v/v) heat-inactivated fetal bovine serum FBS, 100 Unit penicillin/streptomycin for 2 hrs at 37 °C in 5% CO2 incubator and used for further experiments.[66]

6. Cells and Viruses:

The human monocyte cell line U937 (ATCC, USA), Human hepatocyte cell line Huh7 (RRID: CVCL_0336), and monkey kidney cell line Vero (ATCC, USA) were cultured at 37 °C under 5% CO_2 and 70-80% humidity in either RPMI or DMEM (Invitrogen, Sigma Aldrich, St. Louis, USA) along with 10% heat inactivated FBS and 100 U penicillin/streptomycin. The cells were used for performing various experiments. The original cell line is validated and was also Mycoplasma contamination-free. The viruses Dengue virus subtype-2 (DENV2) NGC strain and SARS-CoV-2 (isolate USA-WA-1/2020 from World Reference Center for Emerging Viruses and Arboviruses, from UTMB, Texas, USA) were used for following experiments at MOI of 3 (DENV2) and 0.1(SARS CoV2) respectively. Detail about both viruses is mentioned in our previous work.[66,68]

7. Virus preparation:

DENV2 were propagated in mosquito cell line-C6/36 cells, cultured in L15 media supplemented with 10% FBS and penicillin-streptomycin-glutamine. Cells were infected with DENV2 at MOI 0.1 for 2hrs at 37 °C, 5% CO_2 under continuous gentle rocking. Virus inoculums were prepared in L15 media supplemented with 2% FBS. After removal of the infection, cells were given complete L15 media and kept for 5 days at 37 °C, 5% CO_2. Cell supernatant was then collected, centrifuged at 1000 rpm for 10 mins and purified through

membrane. This purified cell supernatant was aliquoted and kept at -80 °C for further use. The amounts of infectious particles were expressed as focus-forming units (FFU)/mL and titrated in Vero cells.[66]

8. Focus-forming assay:

DENV2 virus stock was serially diluted and infected in Vero cells for 2 hrs. The inoculum was then removed and cells supplemented with fresh media at 37^0C with 5% CO_2 for 4 days. Cells were then fixed with 4% paraformaldehyde followed by permeabilization and blocking. Primary antimouse 4G2 antibody was then added to cells followed by addition of goat antimouse AF488-conjugated secondary antibody. Cells were washed with 1X PBS. Foci were calculated to evaluate the virus titer.[66]

9. Generation of monocytic and liver cell line expressing PHD2 ^{WT}, PHD2 $^{D4E;C127S}$ gene:

In the U937 monocytic cell line and Huh7 liver cell line, the endogenous PHD2/ HIF1α expression was knocked down using liposome-mediated delivery, using shRNA targeting 3' Untranslated region, followed by re-introducing PHD2^{WT} or PHD2$^{D4E;C127S}$ construct into the PHD2- knocked down cells using lentiviral particles.[64]

10. Experimental Design:

Primary Monocytes/U937 cells/Huh7 cells (expressing PHD2 ^{WT,} PHD2 $^{D4E;C127S}$ construct)/ HIF1α Knocked down U937 cells have been kept under Normoxic conditions (21% O_2) and Hypoxic conditions (3% O_2). Cells were incubated under hypoxia for pre-exposure to mimic the high-altitude environment for 8hrs then were infected with DENV2, SARS-CoV-2 at MOI3 (DENV2) and MOI 0.1 (SARS CoV2) for 2hrs and kept for another 14hrs for viral multiplication. Cells and cell supernatants were collected for further analysis.

11. TCN and CAY10585 treatment:

U937 cells having PHD2 ^{WT} and PHD2 $^{D4E;C127S}$ were treated with either TCN (AKT inhibitor) or CAY10585 (HIFα inhibitor) at 5μM or 40μM concentrations for 8hrs before infection and also kept 14hrs post-infection.

12. Real-Time PCR:

Total cellular RNA was isolated from the Primary Monocytes/U937 cells/Huh7 cells (expressing $PHD2^{WT,}$ $PHD2^{D4E;C127S}$ construct)/ HIF1α Knocked down U937 cell pellet using RNAiso Plus reagent (Takara Bio, Japan). cDNA was prepared from 1000 ng of RNA by iScript reverse transcriptase, primers from Super Script four First-strand synthesis system (BIORAD Laboratories USA). 2μL of cDNA from each set is used for qPCR using forward primer and reverse primer with SYBR reagent.[67]

13. Confocal Microscopy:

After completion of experiments, cells were fixed with 3% paraformaldehyde for 20mins at room temperature under normoxic and hypoxic conditions followed by permeabilizing with 0.1% Triton X-100 for 20mins, rocker slow speed. Then blocking was done with 5% Goat Serum for one hour at RT in slow speed rocking condition followed by incubating with primary antibody overnight. Cells were then labelled with Alexa 488 conjugated Secondary antibody. Cells were stained with DAPI for 20 mins at RT followed by mounting with Prolong Gold. Images were captured in a Leica Confocal DMI 6000 TCS-SP8 microscope (Leica Microsystems, Wetzlar, Germany) at 63× oil immersion objective (NA 1.4) Plan Apo objectives and were quantified.[64] Images were captured at 0.50 μm and analyzed by Image J Fiji software.[67] Background density was measured from the area without fluorescence and was deducted from integrated density. Total Fluorescence intensity was measured and calculated from 15 cell images in each set with three individual experiments. Data will be represented as mean ± standard error (SEM).

14. Flow Cytometry:

The infected primary monocytes after completion of the experiment, were first stained anti-CD14 and anti-CD11c, and then intracellular staining was performed. Followed by cells fixation with BD fixation buffer, permeabilized with a permeabilization buffer, and labelled with anti-human HIF1α followed by goat anti-rabbit AF488 secondary antibody. The cells were acquired using a FACS Verse and data were analyzed using FlowJo software.[64,65]

PBMCs from Tibetan individuals were treated with or without SARS-CoV-2 RBD peptide

and kept under normoxia(21%) and hypoxia(3%) for 16hrs. Cells were first stained with CD3 APC, CD4 V450, CD8 PerCP, CD45RO PE-CY-7 followed by fixation with fixative. Further, cells were permeabilized and intracellular IFNγ PE staining was done. Cells were acquired using a FACS Verse and data were analyzed using FlowJo software (Treestar, Ashland, USA).

15. Cytokine Measurement Assay:

Cytokine IFNα was measured by CBA (BD Biosciences, USA.) from the cell supernatant of experimental samples and analyze by FCAP array software (BD Biosciences. San Josh, CA, USA).[65]

16. ELISA:

The expression of IFNβ in experimental cell supernatants was calculated using the commercially available ELISA kit (R & D systems) as per protocol.[66]

17. Immunoblotting:

The intracellular proteins PHD2, HIF1α, P-AKT(Ser473), AKT, P-IRF3, IRF3, α-Tubulin (Cell Signaling Technology, USA.), HIF2α, P-AKT1(Thr308), ACE2, Dengue NS1, and SARS-CoV-2 spike (Invitrogen, USA.) were detected in primary monocytes, U937 monocytic cell line, and Huh7 cell line. Lysis of the cell pellets was done using RIPA along with inhibitor of protease-phosphatase (Sigma, USA) followed by SDS-PAGE.[65]

18. Statistical Analysis:

The Experimental values were calculated from at least three independent experiments and were presented as mean ± SEM (Standard error of the mean). Statistical analysis was performed by either an unpaired t-test or One-way ANOVA followed by Bonferroni post-test if data was distributed normally, paired t-test or Kruskal Wallis test for non-normal distributed data. Graph pad Prism 5.0 software was used to analyze the data and p-values <0.05 were considered as significant.[67]

6. EXPECTED OUTCOME OF THE RESEARCH

Tibetans expressing PHD2 $^{D4E;C127S}$ don not show any physiological response to hypoxia at high altitude (≥2500 m) like acclimatization and mountain sickness. Prolyl hydroxylase 2(PHD2) by binding with oxygen, hydroxylates HIF α subunits generating a binding site for the Von Hippel-Lindau (VHL) protein that results in polyubiquitination and proteasomal degradation under normoxia. Under hypoxia, as PHD2 enzyme cannot bind to enough oxygen, HIF α is stabilized. This results in increase of erythropoiesis and facilitates pathogenesis of Acute Mountain Sickness. The Tibetan haplotype of *EGLN1* expressing PHD2 $^{D4E;C127S}$ has 30% lower Km value for oxygen than PHD2$^{WT.}$ This novel genetic adaptation helps them to adapt in hypoxic environment.

PHD2 is the negative regulator of Hypoxia inducible factors, HIF and HIF is the negative regulator of interferons. Therefore, we hypothesize when PHD2 is active, it degrades HIF subunits, thus interferon response is elevated which helps in reducing the viral infections.

On the hand, PHD2 is also known to directly hydroxylate phosphorylated AKT and inhibit AKT signaling. This Serine-threonine kinase AKT has a key role in promoting viral cell growth, cell proliferation, replication, viral protein translation and inflammation. The suppressed expression of phosphorylated AKT reduces viral replication due to gain of function this Tibetan PHD2 variant under hypoxia. Contrarily elevated phospho-AKT make Tibetan susceptible with PHD2 $^{D4E;C127S}$ variant under normoxic condition.

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